

Selective Measurement of Starch Synthesizing Enzymes in Permeabilized Potato Tuber Slices¹

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ABSTRACT

Osmotically permeabilized potato (*Solanum tuberosum* L.) tuber slices were used to study the biosynthesis of starch under semi *in vivo* conditions. Criteria to distinguish the various enzymes involved in starch biosynthesis were developed based on the characteristics of the enzymes in *in vitro* experiments. Branching enzyme activity was inhibited at pH 8.5 or higher, while the starch synthases functioned optimally between pH 8.8 and 9.1. Unprimed soluble starch synthase activity was only apparent in the presence of sodium citrate (0.4 molar or higher). Granule-bound and primed soluble starch synthase were active in the absence of sodium citrate. Primed soluble starch synthase activity was susceptible to inhibition by 10 millimolar zinc sulfate, while granule-bound starch synthase activity was not. The incorporation of the Glc moiety of ADP-Glc into starch in tissue slices by the various starch synthases was consistent with *in vitro* data with respect to the affinity of the enzymes for substrate, the pH profile, the stimulation by citrate, and the inhibition by zinc sulfate. These data were used to determine the activity of each of the starch synthases in tissue slices: granule-bound and soluble starch synthase transferred 37 and 55 picomoles ADP-Glc per hour per milligram fresh weight into starch of permeabilized tissue slices at 30°C and pH 9.1. In the presence of 0.5 molar sodium citrate, at least 40 picomoles ADP-Glc per hour per milligram fresh weight as transferred into starch by unprimed soluble starch synthase activity.

Most reserve starches of higher plants consist of 20 to 25% amylose and 75 to 80% amylopectin (28). At least three kinds of enzymes are involved in the biosynthesis of both starch components; ADP-Glc synthetase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), and branching (or Q-) enzyme (EC 2.4.1.18).

The first enzyme synthesizes ADP-Glc, which serves as a substrate for starch synthase.

Starch synthase catalyzes the formation of new α -1,4 bonds between preexisting primers and Glc units. This enzyme activity generally occurs in a granule-bound (8, 17, 30) and a soluble form (9, 10, 14), both of which occur as multiple enzymes based on differences in their chromatographic behavior (4, 12, 13, 20, 23, 24). The different isoenzymes are well characterized in maize (20).

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One of the soluble starch synthases (SSS-I) has a relatively high affinity for primer molecules. It elongates both amylopectin and glycogen, its reactivity being higher for amylopectin. Since enzymatic activity is also measurable without exogenously added primer molecules in the presence of at least 0.25 M of sodium citrate or sodium malate (3, 12, 25), the enzyme is referred to as the 'unprimed soluble starch synthase.' The incorporation of ADP-Glc into amylopectin is stimulated fivefold by 0.5 M sodium citrate (20).

The other soluble iso-enzyme (SSS-II, or the primed soluble starch synthase), shows a relatively low affinity for primer molecules, prefers amylopectin to glycogen as a primer, does not exhibit activity without exogenously added primers, and is stimulated by citrate less than twofold when amylopectin is the primer molecule (4, 20, 23, 24).

Branching enzymes hydrolyze α -1,4 linkages and synthesize α -1,6 bonds. Several branching isoenzymes, differing in chromatographic behavior, have been shown to be present in maize (4).

Potato tubers follow the general pattern outlined above. They contain at least one granule-bound starch synthase (15, 30). The existence of multiple granule-bound starch synthases has never been demonstrated since all attempts to isolate native granule-bound starch synthase from potato thus far have failed. Two soluble starch synthases (12) and only one branching enzyme (2, 6) have been identified.

Although there is considerable information on the starch synthesizing enzymes, very little is known about the initiation and elongation of either amylose or amylopectin or about the way in which these polymers assemble into starch granules *in vivo*. Elongation of both starch components has been studied *in vitro* by using (partly) purified enzymes (3, 9, 10, 20, 24, 25). However, since starch synthesis and assembly into starch granules are complex processes, it is difficult to mimic adequately *in vivo* conditions *in vitro*. This difficulty can be circumvented by using an intact *in vivo* system accessible to experimental manipulation such as isolated amyloplasts, isolated protoplasts, or permeabilized tissue slices. We chose to use the latter approach because the isolation of intact and functional protoplasts or amyloplasts is very difficult (11; AS Ponstein, unpublished results) and because the permeabilization of tissue slices entails little or no destruction of the cellular ultrastructure (18).

In the present paper, some of the characteristics of permeabilized potato tuber slices are reported. Since we wanted to

study the specific *in vivo* role of each of the starch synthases, we were interested in obtaining criteria to differentiate between the different enzymes. These criteria were established by comparing the effect of assay conditions and inhibitors on the *in vitro* activities of branching enzyme, granule-bound starch synthase, unprimed soluble starch synthase, and primed soluble starch synthase. The effects of these assay conditions and inhibitors were also determined for the *in vivo* system. These experiments showed that the enzymes involved in the biosynthesis of starch can be measured simultaneously and are distinguishable from each other both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Tubers of a tetraploid variety of potato (*Solanum tuberosum* L., cv Promesse) were purchased from a local farmer. The diploid *amylose-free* potato mutant, recently obtained in our laboratory (15) and the diploid parent strain 7322 were grown in a greenhouse. Potatoes were stored maximally for 6 months at 4 to 8°C. Storage affected the rate of ADP-Glc incorporation into tissue slices (compare Fig. 1, lane A and D) only slightly. Therefore, stored as well as fresh potatoes were used in the experiments described in this paper.

Glycogen and phosphorylase *a* were from Sigma. ADP-[U-¹⁴C]Glc (253 mCi/mmol), UDP-[U-¹⁴C]Glc (313 mCi/mmol), and [U-¹⁴C]Glc-1-P (294 mCi/mmol) were obtained from Amersham, U.K. All other chemicals were of analytical grade.

Isolation of Starch Granules

Starch granules containing granule-bound starch synthase activity were isolated as described before (30).

Isolation of Cell Free Extracts

Potato tubers (100 g) were peeled and homogenized in a Waring Blendor with 100 mL of 100 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 0.1% (w/v) Na₂S₂O₅, and 1 mM DTT for four periods of 15 s separated by 1 min intervals. The suspension was filtered through synthetic cloth and was centrifuged at 25,000g for 40 min. The supernatant was made to 40% saturation with solid (NH₄)₂SO₄. The precipitate was collected by centrifugation (30,000g, 30 min) and was dissolved in 5 to 10 mL of 100 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 1 mM DTT. The solution was dialyzed overnight against the same buffer and was used as the source of soluble starch synthase and branching enzyme activities. All operations were carried out at 4°C.

Assay of Starch Synthases

Granule-bound starch synthase was assayed as described by Vos-Scheperkeuter *et al.* (30), except that the assay was carried out at pH 9.1 instead of pH 8.5.

Primed soluble starch synthase activity was measured as follows: cell free extracts were incubated in 100 μ L assay mixture so that final concentrations were 100 mM Bicine-

NaOH (pH 9.1), 5 mM EDTA, 10 mM GSH, 25 mM potassium acetate, 5 mg/mL glycogen (rabbit liver), 1 mM ADP-Glc, and 0.36 μ M ADP-[¹⁴C]Glc. The reaction was started by adding 10 to 30 μ g protein and was allowed to take place at 30°C for 90 min. The reaction was stopped by adding 2 mL ice-cold methanol-KCl (70% [v/v] methanol containing 1% [w/v] KCl). The tubes were held at 4°C for 5 min or more and were then centrifuged (5 min in a Eppendorf centrifuge). The pellets were dissolved in 150 μ L water and reprecipitated with methanol-KCl to remove noncovalently bound label. The final pellets were dissolved in 300 μ L water and taken up in 2.5 mL emulsifier scintillator 299 (Packard). The amount of incorporated radioactivity was counted in a Packard liquid scintillation counter. Enzymatic activities were calculated based on the specific activity of ADP-Glc and the incorporation of [¹⁴C]Glc and were expressed as percentage of the maximum activity within one set of experiments.

Unprimed starch synthase activity was assayed as above in 100 mM Bicine-NaOH (pH 9.1), 5 mM EDTA, 10 mM GSH, 0.5 mg/mL BSA, 0.5 M sodium citrate, 1 mM ADP-Glc, and 0.36 μ M ADP-[¹⁴C]Glc. After incubation for 90 min at 30°C, the glucans formed were precipitated as above together with 500 μ g glycogen. Enzyme activity was calculated and expressed as above.

Assay of Branching Enzyme and Phosphorylase *a* Activity

Branching enzyme activity was assayed in a Bicine-NaOH buffer rather than in a 100 mM sodium citrate buffer (2, 6) in order to compare inhibition data. Cell free extracts (less than 10 μ g protein) were incubated in a total volume of 100 μ L with (final concentrations) 100 mM Bicine-NaOH, 5 mM EDTA, 10 mM GSH, 25 mM potassium acetate, 1 mM AMP, 0.08 mg/mL phosphorylase *a*, 50 mM Glc-1-P, and 0.17 μ M [¹⁴C]Glc-1-P. After incubation at 30°C for 90 min, the reaction was terminated as described for the unprimed soluble starch synthase.

Phosphorylase *a* activity was determined likewise by replacing the branching enzyme preparation with 50 μ g glycogen. Branching enzyme activities were calculated based on the specific activity of Glc-1-phosphate and the incorporation of [¹⁴C]Glc. Activities were expressed as percentage of the activity of phosphorylase *a* and as percentage of the highest value within one set of experiments.

Preparation of Potato Tissue Slices

Potato tubers were peeled, washed, and cut into halves. Slices of 1 mm thickness were cut from both halves. These were cut into squares of 4 \times 4 mm with a razor blade. The fresh weight of each square was determined (usually 10–15 mg) and each square was incubated in 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) for 60 min, unless stated otherwise, to permeabilize the cells of the tissue slices.

Incorporation of ADP-Glc and UDP-Glc into Starch of Potato Tuber Slices

To study the incorporation of ADP-Glc or UDP-Glc in potato tuber slices, the potassium phosphate buffer was re-

placed by 90 μ L assay-mixture so that final concentrations were approximately 100 mM Bicine-NaOH (pH 9.1), 5 mM EDTA, 10 mM GSH, 25 mM potassium acetate, 0.1 mM ADP-(or UDP-)Glc, and 0.36 μ M ADP(or UDP)-[14 C]Glc. Incorporation of substrate was allowed to take place at 30°C during 90 min. In initial studies the reaction mixture was removed and the squares were washed four times at 30°C for 2 min with 0.5 mL water containing 0.1 mM unlabeled ADP-Glc. Ethanol extracts were made as described by Oparka (22), but less than 1% of the label was recovered in the ethanol extracts. One mL of water was added to the ethanol-extracted tissue slices and they were heated for 2 h in a boiling water bath to gelatinize the starch. Amyloglucosidase (EC 3.2.1.3) at a concentration of 1 mg/mL in 0.1 M acetate buffer (pH 4.6) was added, and the tissue slices were incubated for 2 h at 55°C. The suspension was centrifuged at 14,000g for 30 min, and the radioactivity of both the supernatant and the residue were determined as described above. It appeared that only 1 to 2% of the incorporated radioactivity was insoluble, thus more than 98% of the label was incorporated into starch (22).

Since ethanol soluble sugars were not formed during the incubation with ADP-Glc the washing procedure was simplified to four washes at 30°C of 20 to 25 min each with 0.5 mL 0.2 N HCl. Cell walls were subsequently digested for 15 h according to Hovenkamp-Hermelink *et al.* (15). The amount of radioactivity incorporated was determined as described for the *in vitro* assays.

Inhibition Experiments

Inhibition experiments were performed as described above in assay mixtures containing inhibiting agents. In the case of the *in vivo* inhibition experiments with zinc sulfate, tissue permeabilization was enhanced by adding 5% (v/v) toluene during pretreatment of the tuber squares.

RESULTS

Incorporation of ADP-Glc into Potato Tuber Slices

Freshly harvested potatoes were used to optimize the incorporation of ADP-Glc into tissue slices. Without any pretreatment, 31 ± 6 pmol ADP-Glc was incorporated into tissue slices per h per mg fresh weight (Fig. 1, lane A). Analysis of the labeled material showed that more than 80% of the label was incorporated into amylose and amylopectin (26).

Several treatments to enhance the incorporation of ADP-Glc into tissue slices by permeabilization of the plasma and amyloplast membranes in these slices were tested. Exposure of tissue slices to a hypotonic potassium phosphate buffer, *i.e.* aging (16), gave the best results. The incorporation of ADP-Glc was optimal after aging for 60 min at room temperature and remained constant for at least 2 h. At pH 9.1 and 30°C the incorporation of ADP-Glc into permeabilized tissue slices was linear with time for at least 100 min (data not shown). Per h 92 ± 16 pmol ADP-Glc was incorporated per mg fresh weight (Fig. 1, lane B). Prolonged aging for 24 h resulted in loss of 20% of the maximal amount of ADP-Glc incorporation (data not shown).

The addition of 5% (v/v) toluene to the potassium phosphate buffer (18, 29) or the use of several freeze-thaw cycles

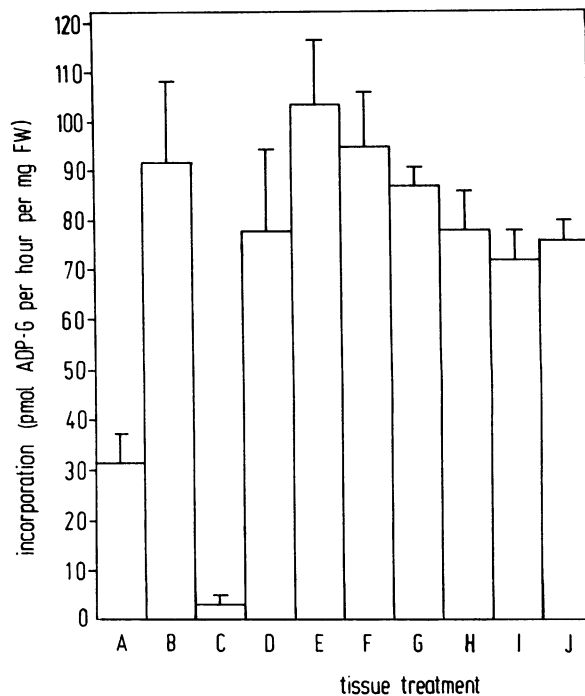


Figure 1. Amount of ADP-Glc incorporated into tissue slices after different procedures of tissue pretreatment. All data were obtained using freshly harvested potatoes, except in lane D where potatoes were stored at 4 to 8°C for 6 months prior to use. Tissue slices were untreated (lane A), or preincubated in 10 mM potassium phosphate buffer (pH 7.0), for either 60 min at room temperature (lane B, D, and E to J), or 2 min at 100°C (lane C). The potassium phosphate buffer was enriched with 0.2% (w/v) sodium dithionite (E) or 1 mM DTT (F). The assays were performed at pH 9.1, 30°C, and 0.1 mM ADP-Glc for 90 min. The incorporation of ADP-Glc in the presence of 1 (lane G) and 10 mM (lane H) G-1-P and 1 (lane I) and 10 mM (lane J) G-6-P is also shown. All values are the mean of three or four experiments (one slice per assay). Standard deviations are indicated.

of the tissue slices (27) did not further increase the incorporation of ADP-Glc into tissue slices.

The incorporation of ADP-Glc was destroyed by heating the tissue squares 2 min at 100°C (Fig. 1, lane C) or by exposing the tissue slices to 0.25 N HCl (data not shown).

Sodium dithionite (0.2% w/v) and DTT (1 mM), often used to stabilize the soluble starch synthases (3, 12, 25), had no effect on ADP-Glc incorporation into tissue slices (Fig. 1, lanes E and F). Addition of 1 and 10 mM G-1-P or G-6-P to the assay mixture did not inhibit ADP-Glc incorporation (*cf.* Fig. 1, lane B with lanes G, H, I, and J). Sucrose (up to 250 mM) did not interfere with the incorporation of ADP-Glc as well. Also, as noted earlier, the slices exposed to ADP-Glc did not produce ethanol soluble sugars. Thus, ADP-Glc is not metabolized prior to incorporation into starch (19, 28).

The above results imply that ADP-Glc diffuses freely across permeabilized plasma and amyloplast membranes to reach the starch synthesizing enzymes, which transfer the Glc moiety of ADP-Glc to amylose and/or amylopectin.

Michaelis Constants of Starch Synthase Activities

The affinities for ADP-Glc of the granule-bound and the soluble primed and unprimed starch synthases were deter-

mined *in vitro* at pH 9.1 and 30°C (Table I). The K_m and V_{max} for the overall incorporation of ADP-Glc into permeabilized tissue slices were also determined. The K_m was comparable to that of the isolated starch synthases, indicating that the intracellular enzymes were sensitive to the extracellular substrate concentration and hence that the tissue slices were effectively permeabilized with respect to ADP-Glc. The V_{max} ranged between 750 and 925 pmol ADP-Glc incorporated per h per mg fresh weight.

High pH Values Eliminate Branching Enzyme Activity

To study the role of each of the different enzymes involved in starch biosynthesis it is necessary to assay the different enzymes separately. Our first goal was specifically to eliminate branching enzyme activity. Only lead acetate (0.6 mM) has been described as an inhibitor of branching enzyme activity (1). However, we were unable to observe inhibition of branching enzyme activity even at 10 mM lead acetate. We therefore tested pH modulation as an alternative approach to differentially inhibit branching enzyme activity.

Figure 2A shows the pH profiles for both of the soluble starch synthases, the granule-bound starch synthase and the branching enzyme as measured *in vitro*. The pH optima of the starch synthases were very much alike: 9.1 for both soluble starch synthase activities and 8.8 for the granule-bound starch synthase. No soluble starch synthase activity could be detected below pH 7.0, but at that pH the granule-bound starch synthase exhibited 60% of its maximum activity. Branching enzyme activity was measured by its stimulation of phosphorylase *a* activity and was optimal at pH 7.8. There was no stimulation of phosphorylase *a* above pH 8.5, while control experiments showed that phosphorylase *a* was still active under these conditions. From this it was concluded that branching enzyme activity was specifically inhibited *in vitro* at pH 8.5 or higher, thus providing a way to eliminate branching enzyme activity during the *in vivo* incorporation of ADP-Glc by the different starch synthases.

The incorporation of ADP-Glc into starch of tissue slices varied with pH and was optimal at pH 9.1 (Fig. 2B). The pH profile found *in vivo* reflects a summation of the pH profiles found for the starch synthases *in vitro*, suggesting that the intracellular enzymes were sensitive to the extracellular pH and hence that the tissue slices were permeable with respect to pH.

Subsequent *in vivo* experiments were carried out at pH 9.1 since branching enzyme activity was specifically inhibited at

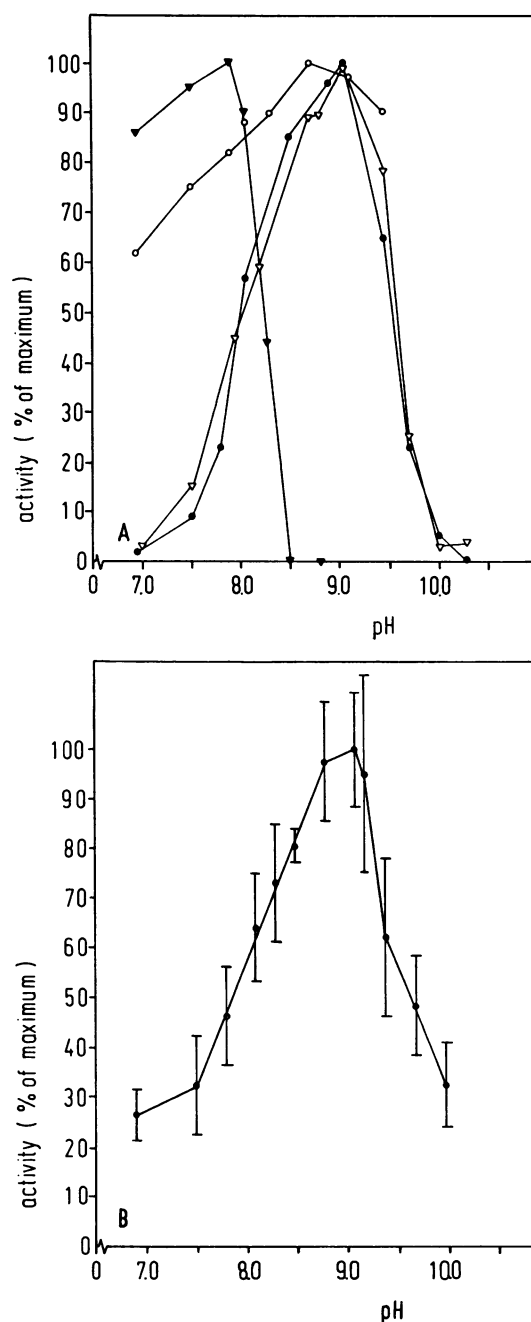


Figure 2. pH dependence of enzymes involved in starch synthesis *in vitro* (A) and *in vivo* (B). All reactions between pH 6.9 and 9.1 were performed in 100 mM Bicine-NaOH buffers, whereas the reactions between pH 9.1 and 10.3 were carried out in 125 mM sodium carbonate-bicarbonate buffers. A, *In vitro* activity of branching enzyme (▲), granule-bound starch synthase (○), primed soluble starch synthase (●), and unprimed soluble starch synthase (▽). The data given are the mean of two assays. B, *In vivo* incorporation of ADP-Glc into potato tuber slices. Data represent three to six experiments, and standard deviations are as indicated.

Table I. Michaelis Constants for Starch Synthases *In Vitro* and *In Vivo*

The Michaelis constants of granule-bound starch synthase, primed and unprimed soluble starch synthase were measured *in vitro* at 30°C and pH 9.1. The affinity for ADP-Glc of total starch synthase activity in permeabilized potato tuber slices was also determined.

Enzyme	K_m
	mM ADP-Glc
Granule-bound starch synthase	1.0
Primed soluble starch synthase	0.8
Unprimed soluble starch synthase	1.2
Tissue slices	0.7–0.9

this pH and starch synthase activity was optimized at the same time.

Unprimed Soluble Starch Synthase Activity Depends on High Amounts of Sodium Citrate

Primed soluble starch synthase activity and granule-bound starch synthase activity are measurable *in vitro* without sodium citrate. In contrast, unprimed soluble starch synthase activity is totally dependent on high amounts of sodium citrate or sodium malate in *in vitro* experiments (3, 12, 20, 23, 25). In case of potato, at least 0.4 M sodium citrate was necessary to stimulate the unprimed soluble starch synthase activity *in vitro* (Fig. 3A).

The *in vitro* effect of sodium citrate on granule-bound and primed soluble starch synthase activity was also measured (Fig. 3A). Primed soluble starch synthase activity was stimulated by 0.2 M sodium citrate and slightly inhibited by higher amounts of sodium citrate. Comparable curves were found when amylose and amylopectin primed the reaction instead of glycogen (data not shown). The granule-bound starch synthase was slightly inhibited by increasing amounts of sodium citrate (Fig. 3A).

The effect of sodium citrate on the *in vivo* incorporation of ADP-Glc into starch of potato tuber tissue slices was biphasic (Fig. 3B). The curve resembles a summation of the curves found for the soluble starch synthases and the granule-bound starch synthase (Fig. 3A). Thus, the stimulating effect of 0.5 M sodium citrate could be recognized. This was interpreted as representing the stimulation of unprimed soluble starch synthase activity under *in vivo* conditions.

Specific Inhibition of Primed Soluble Starch Synthase Activity

In tissue slices, granule-bound and primed soluble starch synthase activity were always assayed together at pH 9.1 and 30°C. These two synthases can, in principle, be differentiated, based on their substrate specificities (8, 9). However, there was very little incorporation of UDP-Glc into starch of tissue slices (approximately 2 pmol/h/mg fresh weight), and thus, in practice, substrate specificity could not be used to differentiate between the two starch synthase activities *in vivo*.

Since the pH profiles of the starch synthases were similar (Fig. 2A), enzymatic inhibitors had to be used. The potential of several inhibitors in differentiating between granule-bound and soluble starch synthase activities was examined *in vitro*. Table II summarizes the inhibition data obtained.

pCMB destroyed both soluble starch synthase activities. It also affected granule-bound starch synthase activity at concentrations which did not inhibit the soluble starch synthases completely, thereby complicating the interpretation of *in vivo* inhibition data by pCMB.

Phenolic compounds which have been found to inhibit granule-bound starch synthase activity (8) were tested as inhibitors of soluble starch synthase activities. In three cases tested (phenol, saligenin, and hydroquinone) the soluble starch synthases were more readily inhibited than the granule-bound starch synthase. The inhibition of the soluble starch

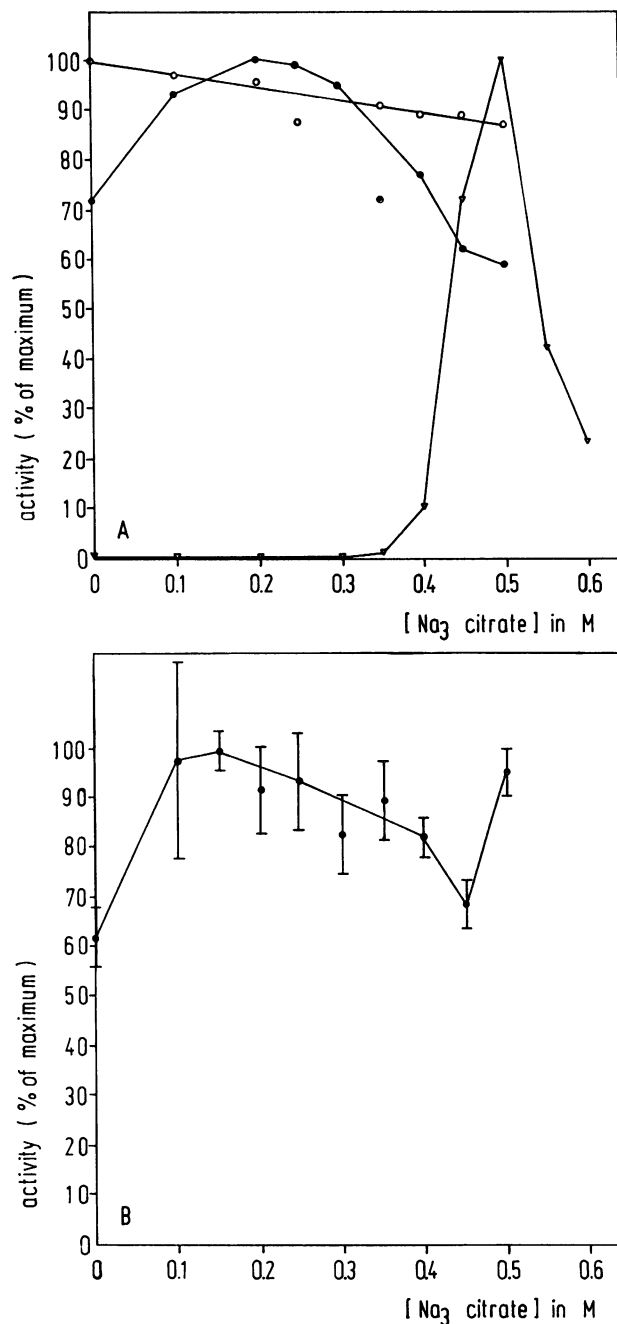


Figure 3. Effect of sodium citrate on the activity of starch synthesizing enzymes *in vitro* (A) and *in vivo* (B). Incubations were as described in the section "Materials and Methods" except for the addition of different amounts of sodium citrate. A, *In vitro* incorporation of ADP-Glc by granule-bound starch synthase (○), primed soluble starch synthase (●), and unprimed soluble starch synthase (▽). Data are the mean of two assays. B, *In vivo* incorporation of ADP-Glc into potato tuber slices. Data represent the mean of three to six experiments. Standard deviations are indicated.

Table II. Effect of Several Inhibitors on the Activity of Granule-Bound Starch Synthase (GBSS), Primed Soluble Starch Synthase (SSS-A), and Unprimed Soluble Starch Synthase SSS-B)

Enzymatic activities were determined at pH 9.1 and 30°C with and without the inhibiting agents. All values are the means of two or more assays.

Additions	Inhibition		
	GBSS	SSS-A	SSS-B
		%	
5 mM pCMB	0	74	83
10 mM manganese chloride	0	29	? ^a
10 mM zinc sulfate	0	95	?
20 mM ammonium heptamolybdate	63	100	?
5% (v/v) toluene	0	0	N.D. ^b
2% (v/v) phenol	87	100	100
30% (v/v) saligenin ^c	5	49	N.D.
40% (v/v) hydroquinone ^c	69	100	N.D.

^a Not measurable (see text). ^b Not determined. ^c Saturated solutions in water at 4°C.

synthases was again incomplete at concentrations that already affected the activity of the granule-bound starch synthase.

Metal ions such as ammonium heptamolybdate, manganese chloride, and zinc sulfate inhibited primed soluble starch synthase activity readily. Zinc sulfate at 10 mM inhibited 95% of the primed soluble starch synthase activity but left granule-bound starch synthase activity unchanged (Fig. 4A).

Specific Inhibition of Primed Soluble Starch Synthase Activity in Wild Type and Amylose-free Tissue Slices

Zinc sulfate was used to inhibit specifically primed soluble starch synthase activity in *in vivo* experiments. When permeabilized (wild type) tissue slices were exposed to increasing amounts of zinc sulfate some inhibition of ADP-Glc incorporation into tissue slices was observed. However, the extent of the inhibition varied and was not reproducible (data not shown).

When 5% (v/v) toluene was added to hypotonic permeabilizing buffer, reproducible results were obtained. The total incorporation of ADP-Glc into starch was unaffected by 5% toluene (data not shown), but increasing the zinc sulfate concentration from 0 to 10 mM resulted in a decrease of ADP-Glc incorporation into tissue slices (Fig. 4B). The inhibition profile resembles the profile shown for primed soluble starch synthase activity, except that *in vivo* approximately 40% of the activity was left uninhibited.

Permeabilized tissue slices from amylose-free potato tuber tissue (15) showed an incorporation rate of 57 ± 7 pmol ADP-Glc/h/mg fresh weight. The incorporation was almost completely (92%) destroyed in the presence of 10 mM zinc sulfate.

DISCUSSION

Both starch components, amylose and amylopectin, are synthesized in amyloplasts by granule-bound and soluble starch synthases in concert with branching enzyme(s) (3, 4, 15, 25). The interaction of different starch synthases and

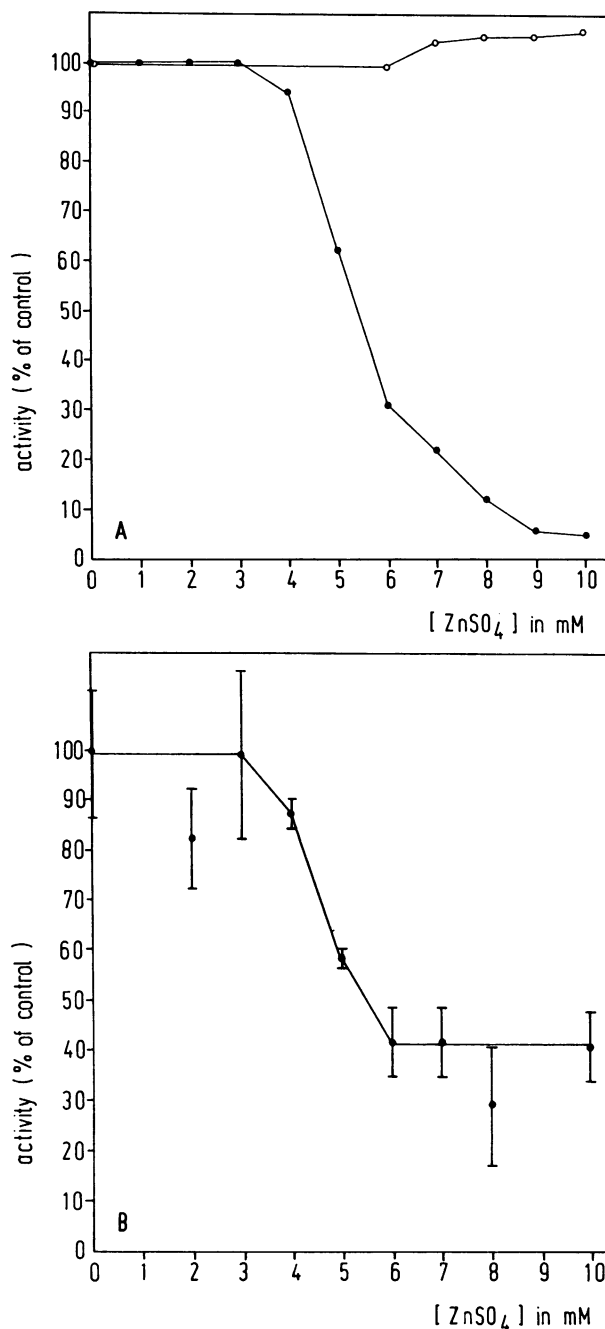


Figure 4. Effect of zinc sulfate on the activity of starch synthesizing enzymes *in vitro* (A) and *in vivo* (B). Assays were carried out as described in the section "Materials and Methods". A, *In vitro* incorporation of ADP-Glc by granule-bound starch synthase (○) and primed soluble starch synthase (●). Data are the mean of two or three assays. B, *In vivo* incorporation of ADP-Glc into potato tuber slices. Tissue slices were permeabilized in potassium phosphate buffer (pH 7.0), containing 5% (v/v) toluene. Data are the mean of two to four experiments. Standard deviations are as indicated.

branching enzymes in the process of starch biosynthesis are most often studied *in vitro* using (partly) purified enzymes (3, 25). However, it is difficult to determine whether, and to what extent, such experiments reflect the functioning of the starch

synthesizing machinery *in vivo* and it is desirable therefore to study starch synthesis under semi *in vivo* conditions. One approach is to use isolated amyloplasts (11, 19) for such studies. Despite repeated attempts, we have not been able to prepare intact, functional amyloplasts from potato tissue. We therefore turned to permeabilized cells as a possible system in which to study starch synthesis and granule assembly. Permeabilized cells have been useful in the study of DNA and cell wall synthesis in bacteria and eucaryotes (7). In the present paper, we show that it is possible to treat potato tissue slices such that cellular plasma and amyloplast membranes are rendered sufficiently permeable to permit access of substrates to starch synthesizing enzymes.

The best procedure for rendering starch synthesizing enzymes accessible to ADP-Glc was to expose potato tissue slices to a low ionic strength buffer for one h. The synthesis of starch by such tissue slices was studied.

Incorporation of ADP-Glc into Starch by Potato Tissue Slices

ADP-Glc is incorporated into potato starch by at least two soluble starch synthases showing primed and unprimed activities (12), one granule-bound starch synthase (15, 30) and one branching enzyme (2, 6). The different pH optima of starch synthases and branching enzyme (Fig. 2A), the citrate requirement of unprimed soluble starch synthase activity (Fig. 3A), and the insensitivity to zinc sulfate inhibition of granule-bound starch synthase activity (Fig. 4A) could be used to differentiate these enzymes. Moreover, the availability of an *amylose-free* potato mutant allowed the study of starch synthesis in tissue slices which lack the granule-bound starch synthase (15).

The characteristics of starch synthesis by permeabilized tissue slices (K_m , pH profile, and sodium citrate stimulation) were consistent with the characteristics of the isolated starch synthases (Figs. 2, 3, 4; Table I). Only the sensitivity to zinc sulfate of primed soluble starch synthase activity was not observed in permeabilized tissue slices. However, zinc sulfate inhibition of soluble starch synthesis in tissue slices could be observed after toluene treatment, suggesting that toluene disrupted intracellular structures, possibly membranes or protein-lipid complexes (5), which apparently shield the primed soluble starch synthase from zinc sulfate.

Relative Contributions of the Starch Synthases to the Total Incorporation of ADP-Glc by Tissue Slices

Based on the differing characteristics of the starch synthases, their activities in permeabilized tissue slices could be differentiated by manipulating the buffer composition used in ADP-Glc incorporation experiments (Figs. 2, 3, 4). At pH 9.1 and 30°C, 92 ± 16 pmol ADP-Glc/h/mg fresh weight was incorporated into wild type tissue slices. This was the result of granule-bound starch synthase and primed soluble starch synthase activity (Fig. 4). In the presence of 10 mM zinc sulfate, 37 pmol ADP-Glc was incorporated per h per mg fresh weight. This was the result of granule-bound starch synthase only (Fig. 4). Consequently, about 55 pmol ADP-Glc/h/mg fresh weight was incorporated by the primed solu-

ble starch synthase activity. This corresponds well with the activity of primed soluble starch synthase measured in *amylose-free* tissue slices (57 ± 7 pmol ADP-Glc/h/mg fresh weight).

The ratio of granule-bound versus primed soluble starch synthase activity *in vivo* (0.7) is difficult to compare with the ratio obtained from *in vitro* experiments, since this ratio depends heavily on the extraction buffer being used (5, 13).

Unprimed soluble starch synthase activity catalyzed the incorporation of at least 40 pmol ADP-Glc/h/mg fresh weight (the stimulation by sodium citrate shown in Fig. 3B). The ratio of primed versus unprimed soluble starch synthase activity *in vivo* (1.4) was lower than the ratio found in crude potato extracts by us (3.7–4.0) and others (4.1, Ref. 12). This is due to the fact that unprimed soluble starch synthase activity disappears from crude extracts within 2 d, while primed soluble starch synthase activity gradually disappears in 2 months (our unpublished results). Thus, the ratio of primed to unprimed soluble starch synthase activity is more or less unaffected in tissue slices (measurements performed within 3 h after preparation), whereas the ratio increases in crude extracts (measurements performed several hours after preparation).

Starch Synthase Rates in Potato Tuber Slices Compared to the Rate Found in the Field

Morrell and ap Rees (21) have estimated that growing potato tubers accumulate starch with a rate of about 2.4 nmol Glc/h/mg fresh weight. Tissue slices from mature potato tubers showed a V_{max} for Glc incorporation from ADP-Glc of about 0.8 nmol/h/mg fresh weight.

The V_{max} estimated for starch synthesis in tissue slices from mature potatoes is not strictly comparable to the rate of starch synthesis in growing potatoes since starch accumulation is more rapid in growing than in mature potato tubers (14). Moreover, the optimal conditions found in our experiments on starch synthesis in tissue slices (pH 9.1, 30°C, permeabilized membranes and the loss of starch granules and soluble enzymes from the outer cut cells) differ from those which apply to growing potatoes. Nevertheless, although the rate of starch synthesis by tissue slices and growing potatoes cannot be compared directly it is clear that these rates are of the same order of magnitude. This suggests that the starch synthesis machinery in tissue slices remains relatively intact during the permeabilizing procedure described in this paper.

With this system it is possible, therefore, to study the synthesis of amylose and amylopectin, under *in vivo* conditions, since all groups of starch synthase activities can be distinguished in potato tuber slices. We expect that this system will be useful in identifying the molecules that prime the different starch synthases *in vivo* as well as in further studying the processes which lead to the production of amylose and amylopectin and their assembly into starch granules.

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